REFLECTIONS

Stents, Statins, and Other Dumb Luck

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In reflecting on the past 80 years, at least as they have affected me, it seems that fate (or nature or God, take your choice) has dealt me an unusually good hand. For example, my father and brother both had serious coronary heart attacks in their early 50s. While I was developing the same blocked arteries, methods were being devised to treat the condition with stents and statins. Much of my good fortune has been dumb luck like this, simply being in the right place at the right time. I know it is hyperbole, but growing up in the fields of biochemistry and biology has been a little like growing up as a musician in 18th century and early 19th century Vienna. An entire area of human endeavor has rolled out like a carpet in front of me. I have seen and understood and even have had a small part in it. I have worked with great men, Fritz Lipmann and Salvador Luria, to name just two, and was in the department of biology at the Massachusetts Institute of Technology (MIT) for most of the 40 years as it developed. Now, I am with a group of great colleagues and close friends in a small department in the Henry M. Goldman School of Dental Medicine at Boston University. I plan to retire soon but, to tell the truth, would not even mind if, by chance, I should leave my office feet first!

I was undecided about career direction until, as a senior in West Springfield (Massachusetts) High School, I took a course in physics. The teacher was a disorganized, little old man with a crooked tie. He was a poor teacher, but it did not matter a bit. As soon as I opened the textbook, a miracle appeared. I had enjoyed math courses, but, to my amazement, I found that the physical world could be understood and analyzed with algebra. I waffled for a while during this period by feeling I could use science and still continue my family's multigeneration Methodist tradition by becoming a medical missionary. When I was thinking about this medical missionary option, I did, in fact, at least partly believe in it. For better or worse, it kept me out of the military draft (I will let you figure out the draft for which war) in progress at the time, but that was not my intent. In any case, by the time I hit organic chemistry at DePauw University in Indiana, I realized that science, and specifically biochemistry, was what I wanted to do.

For my generation, as well as several before and after mine (in some way, this seems to be an American phenomenon), science was not a vocation like law or accounting but was a way of life. An illustration of what this means is given by Linus Pauling in his description, paraphrased below, of the Nobel Prize-related conception of the α-helix.

> Visiting at Oxford, I caught cold and was required to stay in bed for about three days. After two days, I got tired of reading detective stories and science fiction, and I began to think about the problem of the structure of proteins . . . I realized concerning the structures of amino acids and peptides determined by Dr. Corey and others that there had been no surprises whatever. Bond angles, etc. conformed to the values I had already formulated in 1937 . . . As I lay there in bed, I had an idea of a new way of attacking the problem. Back in 1937, I had been so impressed with the fact that the amino acids in any position in the polypeptide chain may be any one of 20 different kinds that, with respect to folding, they might be nearly equivalent had never occurred to me. I asked my wife to bring me pencil, paper and a ruler. By sketching a polypeptide chain on a piece of paper and folding it along parallel lines, I succeeded in finding two structures that satisfied the assumption about equivalence. One of the structures was the alpha helix.

I remember that, during days off on excursions into the countryside, my DePauw first-year organic chemistry course buddies and I spent much of our time talking organic reactions and reaction mechanisms. This was not to impress anyone but was just the output of our streams of consciousness. This American "nerdiness" has many consequences, not the least of which was the lead the United States established in areas of science and technology.

My commitment to a career in a specific area of science came with the organic chemistry course during my junior year at DePauw. The professor, Howard Burkett, was an energetic, upbeat Midwesterner who enjoyed interaction with students. Exactly what had to be covered was outlined in his notes (in 1950, organic chemistry was a semi-empirical subject that involved getting a feeling for the kinds of covalent bonds the carbon atom can form), but he would chat with the students on anything and everything until it became obvious that he would need every second of remaining time to get through the day's outline. I remember sitting in the library early on a beautiful sunny Saturday afternoon feeling that I did not understand anything at all about organic chemistry, when suddenly, in a flash, the whole thing became clear. I realized that one had to learn the nuances of the various types of carbon-X covalent bonds. Given a feeling for ease of formation, stability, etc., one could put together an unlimited variety of compounds (most of which had never been seen on the face of the Earth). I knew now what I wanted to do because, in addition to the fascination with the subject itself, I also was aware that the major building blocks of biology (amino acids, carbohydrates, nucleotides, and lipids) were specific varieties of organic chemicals. It seemed hard for me to understand why everyone did not realize that biochemistry must be the most intensely interesting subject in the world.

This entry into biochemistry by way of organic chemistry led to my becoming a graduate student with H. E. Carter in the chemistry department at the University of Illinois at Urbana-Champaign, where I also had contact with the outstanding microbiology department that included Luria, Irwin C. Gunsalus, and Sol Spiegelman. The latter group made me realize that biology does not exist to keep chemists entertained. Chemistry is only a necessary tool for understanding biology.

When I arrived in Urbana-Champaign (to sleep in a sweltering attic room during the summer of 1952), the Carter group was working in two areas: the isolation of new antibiotics from *Streptomyces* cultures and the isolation and structural analysis of sphingolipids. One observation made by the group isolating sphingolipids from wheat flour was surprising and should have made me think (in fact, it should probably have scared me to death). When elementary analysis was carried out on the crude lipid mixture, it was found that 15% of the weight of the material could not be accounted for. After much head-scratching, the realization dawned, and was confirmed, that this 15% represented covalently bound chlorine introduced into the lipids of flour during the bleaching process. Thus, it was clear that hundreds of new compounds were being created and introduced into the food supply by bleaching. The search for the trace, or not so trace, compounds in our environment that are responsible for breast cancer and other ills is truly a search for a needle in a haystack. At the time, W. C. Rose also was working on the amino acid requirements of man. Any current human use committee would be shocked to learn that the lack of L-valine, especially when reinforced by the addition of D-valine to the diet, could lead not only to drastic weight loss but also to mental problems, episodes that cleared but were still worrisome. In fact, the human studies showed only that our amino acid needs are the same as those of the rat, except that our intestinal bacteria will satisfy the need for histidine.

The Carter group had close connections with the pharmaceutical industry, which was just beginning to realize the commercial potential of the burgeoning field of metabolic biochemistry. This connection turned out to be a mixed blessing. They provided me with a generous stipend and also ran what seemed to be a really interesting cooperative group made up of Lilly, Upjohn Co., Parke-Davis, and the University of Illinois biochemists. The group combined forces to screen bacterial cultures isolated from soil for antibiotics. The screening quickly resulted in the isolation of a number of leads, and we were able to see the whole operation as it developed. What eventually happened, however, was that, as soon as really exciting compounds appeared on the horizon, the race for exclusive patents led to acrimonious fights for exclusive control rather than cooperation. The only thing that seems strange now is that this inevitable outcome was not seen from the beginning when the "Midwestern Group" was first formed.

One of the compounds that caused the breakup was chloramphenicol, which had, of course, been isolated at Parke-Davis in 1947. However, the Urbana-Champaign group felt they had some kind of claim on the substance. In any case, everyone agreed that chloramphenicol was a simple, interesting compound, so why not see whether the new science of tracing metabolic pathways could not be

applied to it, and why not assign the job to the eager new graduate from DePauw? We carried out the bacterial growth work and feeding of radioactive compounds to the cultures in the plant pathology laboratories and the isolation and analysis of the radioactive chloramphenicol in the posh new quarters of the radiocarbon laboratory, which had recently been established at the university for Robert Nystrom. The final pieces of the puzzle came together when numerous ^{14}C-labeled compounds, including phenylalanine, became available from commercial sources. Phenylalanine was of special interest because the addition of the compound to growth medium stimulated chloramphenicol production. Because phenylalanine and chloramphenicol have the same carbon skeleton, it was easy to jump to the erroneous conclusion that the amino acid was being used as a direct biosynthetic precursor for the antibiotic.

To make a long story short, the labeling experiments with phenylalanine indeed showed incorporation into chloramphenicol, but all of the label was in the dichloroacetic acid portion of the molecule! Clearly, phenylalanine was being degraded to general precursors of some kind, with no incorporation of the ring and side chain into the ring and side chain of the antibiotic. An even bigger surprise awaited me when I looked into the metabolism of *p*-nitrophenylserinol, the complete backbone of the antibiotic without the dichloroacetic acid attached. Because adding this backbone structure to the growth medium of the organism produced a huge increase in antibiotic production, it seemed certain that the beast simply added dichloroacetic acid to the core. Wrong again! When I looked into it, I found that there was no incorporation of *p*-nitrophenylserinol into chloramphenicol at all and that the bug simply acetylated the added compound to form *N*-acetyl-*p*-nitrophenylserinol (1), which happens also to be an antibiotic, although a somewhat less potent one per milligram than chloramphenicol. Later work by others showed that chloramphenicol is made by a pathway similar to the aromatic amino acid synthesis pathways but one that is completely independent beyond the chorismate stage (2). My thesis work confirmed my love of biochemistry and taught the lesson (in spades) never jump to conclusions even where they seem obvious. If you need to know, get the data!

Sulfate Activation

I did my postdoctoral research at Massachusetts General Hospital (MGH) in Boston starting in 1955 with Fritz Lipmann, the great German biochemist. Lipmann was trained as an M.D. but became fascinated with concepts of biological energy metabolism, especially oxidative phosphorylation. Rather than simply defining the structures of metabolic intermediates and possible pathways, Lipmann was looking for general principles of biological energy conversion. He was a gentle, soft-spoken man who had more profound thoughts and insights than anyone I have ever known. All the time I knew him, I felt I was in the presence of a great man. In passing, I should mention that, according to rumor, Lipmann was the only person ever promoted directly from instructor to full professor at Harvard Medical School. He emigrated from Germany to Boston before World War II and found a spot in surgery at MGH but was not well known in the Harvard community. At some point, however, the biochemistry department got wind of the Nobel Prize that was about to be awarded to Fritz and acted accordingly. The award was for discovery of coenzyme A and for Lipmann's great essay on the concept that ATP is the common energy currency of the cell. It is mind-boggling to think how far biochemistry and molecular biology have come in less than 50 years! I began graduate school the year Watson and Crick published their *Nature* paper on the structure of DNA. Even five years later, messenger RNA was a foggy concept. Biochemistry was a fairly well defined discipline, but essentially all of molecular biology has developed since then.

When I arrived at MGH to start my postdoctoral research in 1955, Lipmann was extending his interest in the way ATP was used in group activation, and he put me to work on a system described by R. H. DeMeio that involved the activation and transfer of sulfate ion. The activating enzymes are present in liver and yeast, so the problem was one of enzymology and nucleotide purification. The phosphosulfate anhydride bond is thermodynamically far uphill from ATP, so both products of the initial reaction need to be removed to reach any reasonable yield of mixed anhydride. Just as Lipmann had predicted, the thermodynamic potential energy for the process all came directly from ATP. In my structural and enzymatic studies, I showed that the molecule is made by an initial displacement of pyrophosphate with sulfate, followed by hydrolysis of inorganic pyrophosphate. A second molecule of ATP is then used to remove the initial product of the reaction by phosphorylation of the 3'-position (3). Lipmann explored a number of other systems over the years, and his biggest disappointment came with the discovery that oxidative phosphorylation does not involve covalent intermediates. In any case, the frantic search for covalent intermediates in oxidative phosphorylation in the Lipmann laboratory did lead to the discovery of carbamyl phosphate. At about this time, our group transferred from

MGH to The Rockefeller University, and I began to think about an independent appointment.

Phage Conversion and Bactoprenol

In 1960, I moved on to MIT and, within six months, became fascinated with the phage conversion phenomenon that was being studied by Salvador Luria and his group. Salva was a marvelous person with a wide range of interests. As well as being a Nobel Prize microbiologist and one of the founders of the American Society of Microbiology, he was well informed in art, literature, and philosophy. The discovery that the Luria group had made that turned me on was that the antigenic structure of *Salmonella* lipopolysaccharide could be controlled by temperate or lysogenic bacteriophages. The concept of control of a cellular characteristic by a virus was new and remarkable. In this case, the structure of LPS appeared to be totally dependent on the presence or absence of two viruses in the cell. This has obvious biological significance because many viruses that attack Gram-negative bacteria start infection by binding to the LPS outer membrane. After solving the structure of the LPS in the starting and "converted" cells (4), we set up an *in vitro* system for assembly of the O-antigen polysaccharide chains, starting with sugar nucleotide precursors, and got excited when we found that the repeating units of the polysaccharide were preassembled on a lipid carrier (5). The "we" included Rich Losick, Dennis Bray from England, Andrew Wright from Scotland, and Marcello Dankert from the Luis Leloir laboratory in Argentina.

The structure of the lipid carrier was a puzzle that was solved by isolation and analysis. The linkage between the oligosaccharide and lipid was acid-labile, and the correct answer was reinforced when I heard Konrad Bloch complaining about the acid lability of isopentenyl pyrophosphate. All of our data had suggested a polyisoprenoid, and that is what it turned out to be (6)! When we had solved the structure with the help of mass spectrometry, Marcello proposed to name the lipid in my honor using the name of the North American Robin *Turdus migratorius*. When he suggested "terditol," I thanked him but declined the honor. We settled on ACL for antigen carrier lipid. Later, the name was changed to bactoprenol. When we had the structure of the intermediate, we guessed at, and then showed, the cycle involved in polysaccharide assembly.

What about the viral genes that alter the structure of the O-antigen? It is interesting that the phage does not alter the structure of the lipid-linked intermediate but redirects the polymerase reaction. The cellular α-polymerase is shut down rapidly and is replaced with a phage-specified polymerase that catalyzes the formation of β-linkages between the subunits. The phage also shuts down a cellular O-antigen transacetylase. We still do not completely understand the inhibitory systems, and the polymerases have never been compared. The reason that the phage carries these genes is equally mysterious, but the pathogenicity of *Salmonella* may be affected by changes in LPS antigenicity, and the phage also prevents superinfection by shutting off its own receptor.

During the 1960s, I went back to Rockefeller twice a year to spend a day talking with Lipmann and his group. At the time, Lipmann was involved in investigating mechanisms of protein synthesis. When I showed him the LPS synthesis cycle, he immediately jumped to the conclusion that the polysaccharide chains grow just as protein chains grow, by adding the activated growing chain to the new incoming subunit. This was heresy to carbohydrate biochemists because it meant that the chains grow at their reducing ends rather than at their nonreducing ends as starch and glycogen do. As usual, Lipmann was right, and accepted dogma was wrong. A few pulse-chase experiments showed us that the chains do, indeed, grow at the reducing ends (7).

Asparagine-linked Glycosylation

For me, it was just a short hop from the bacterial O-antigen problem to eukaryotic asparagine-linked glycosylation. When we started our studies, the mammalian *N*-linked glycosylation field was already well developed by William J. Lennarz, Stuart Kornfeld, Robert G. Spiro, and others. I do not have space to review the history, but I should at least clarify the record about the discovery and characterization of the mammalian dolichol-linked oligosaccharide. The Spiro group was the first to isolate the lipid-linked oligosaccharide and show that it contained glucose as well as mannose. They showed that the oligosaccharide was linked by a pyrophosphate bridge to dolichol, and with pulse-chase and inhibitor experiments, they were the first to show that the oligosaccharide could be transferred intact to protein. Starting from this point, it was easy for the rest of us to take the next step and suggest that the dolichol-linked oligosaccharide is the universal precursor of all asparagine-linked oligosaccharides (8). Finding the genes and enzymes responsible for making the 14-sugar dolichol-linked precursor started with second-year graduate student Tim Huffaker's suggestion to me that he would incorporate high levels of radioactive mannose into yeast cells, freeze them away for a month or two to let the wild-type cells die of radiation damage, and then recover mutants in the *N*-linked glycosylation pathway that had incorporated less radioactivity. Miraculously, this strategy worked like a charm (9)! Further work on the

pathway enzymes is a well known story and has been reviewed in a number of places, as have the enzymology and genetics of the Golgi processing enzymes. However, exciting new aspects are still being discovered, such as the mechanism of "flipping" of the five-mannose dolichol-linked intermediate from the cytoplasmic to the luminal face of the endoplasmic reticulum.

Chitin Synthesis

To continue the story, in 1983, my close friend Victor Ginsberg persuaded me to spend a sabbatical leave at the National Institutes of Health. I had plans to spend time on several projects, proteoglycan synthesis with Vince Hascall and bacterial capsule synthesis with Willie Vann to mention just two. But, just for the fun of it, I wanted to spend some time with Enrico Cabib and Marty Slater on the chitin synthesis problem. When there is a choice between things you probably should do and something that looks like real fun, I do not need to tell you what happens.

Enrico had beautifully characterized a membrane-bound enzymatic activity in yeast that required proteolytic activation, and Marty had isolated mutants that lacked chitin synthase activity *in vitro*. Given the exquisite localization of chitin fibers in yeast, here was a great problem in cell biology with all the tools ready to analyze it. The enzyme, a plasma membrane-bound zymogen, made chitin chains *in vitro* from UDP-GlcNAc after treatment with protease. Mohinder Kang had just finished purifying the protein when I arrived on the scene. Our first hypothesis was that the zymogen was present throughout the plasma membrane and was activated by proteolysis in the bud-neck region during the cell division process. Therefore, we felt we should look for specific proteolysis in the bud-neck ring. What did we do? As a first step, we cloned the gene, knocked it out, and voilà! No, not voilà because, in the knock-out, chitin synthesis and ring formation *in vivo* were still normal (10). When we found and knocked out a second gene and the level of chitin in the cells actually went *up*, we became convinced that reverse genetics was taking us in reverse!

To summarize the final answer, Enrico's group, along with Christine Bulawa and Peter Orlean in my laboratory, showed the following. (*a*) Chitin synthases are membrane-bound enzymes that polymerize GlcNAc residues from the nucleotide sugar and deposit chitin or extrude it through the plasma membrane. The only mechanism that makes sense is extrusion of nascent chains, followed by spontaneous "crystallization" after extrusion. (*b*) Each of the chitin synthases (all *three* of them) has a separate gene, a separate function, and a separate location in the cell and

is under separate regulatory control. In other fungi, there are as many as seven or eight enzymes, all with more or less identical catalytic function (11). (*c*) In *Saccharomyces*, Chs3p (obviously the last to be found) makes 90% of the chitin in the cell and has three functions. It makes chitin in the bud-neck ring, it deposits extra chitin in the lateral wall when the cell wall is under stress, and it works with chitin deacetylase and other proteins to form chitosan in sporulating cells. There are intracellular vesicles called "chitosomes" that carry Chs3p to the bud-neck region as it forms and to the cell surface to deposit extra chitin when the cell wall stress response is turned on. Work on these interesting problems obviously continues.

Protist *N*-Linked Glycosylation

One of the aspects of the asparagine-linked glycosylation cycle that I found interesting was its near-identity in organisms as diverse as animals, fungi, and plants. It must have existed in the "ur-eukaryote" at the beginning of multicellular evolutionary development. Because this is the case, John Samuelson and I were not optimists when we started to explore the cycle in the protist world. A distinguished parasitologist, John had recently been involved in a major way in sequencing the parasitic protists when he joined our department of molecular and cell biology at Boston University. To our surprise, we found that Alg (asparagine-linked glycosylation) genes and enzymes are missing in sets from each of five different groups of parasitic organisms and, in fact, that all the enzymes that add glucose and mannose to the dolichol intermediate are missing from *Giardia* and *Plasmodium*, organisms that had previously been thought to lack asparagine glycosylation. Furthermore, a careful analysis showed that the present diversity of protist and fungal dolichol-linked glycans does, in fact, appear to result from secondary loss of glycosyltransferases from our proposed ur-eukaryote (12). It clearly *did* contain the complete set of dolichol-linked intermediates that we find today.

Epilogue

Where do I/we go from here? It is probably silly to even speculate. As John Updike says in his introduction to *Hugging the Shore*, "At all times, an old world is collapsing and a new world arising; we have better eyes for the collapse than the rise, for the old one is the world we know." New science is rising all the time, and my only hope is to keep up with it as best I can. What I can do is state my general philosophy of science and my appreciation of mentors, friends, and colleagues. My philosophy is simple; it is borrowed directly from Lipmann, who said, in his Nobel Prize essay, "The purpose of scientists often may be none but

just to push back a little the limits of comprehension. Their findings mostly have to be expressed in a scientific language that is understood only by a few. We feel, nevertheless, that the drive and urge to explore nature in all its facets is one of the most important functions of humanity."

I have already expressed my appreciation for the great men I have considered mentors, Fritz Lipmann and Salva Luria, but other mentors have been almost as important. As mentioned, I would not have become a biochemist without that junior level organic chemistry course taught by Howard Burkett at DePauw. In my inner ear, I even sometimes hear the dictum of my junior high school English teacher, who told us, "Never write to be 'understood.' You must always write so you cannot possibly be misunderstood." There was also Herb Carter, who, even as he was becoming head of the chemistry department at Urbana-Champaign, still took the time to see that I was working in a productive way on my thesis problem. I also know that he, Salva, and Gunsalus helped to arrange my difficult-to-get postdoctoral position with Fritz, who had just received the Nobel Prize and had innumerable others knocking on his door.

Finally, let me say that one can never adequately thank friends and family for their help and support. As already mentioned, I am now a member of a small department of great colleagues and close friends in the Henry M. Goldman School of Dental Medicine at Boston University. I have known Carlos Hirschberg, who discovered and developed the field of nucleotide sugar transport, for a long time. He was a postdoctoral fellow in my group at MIT and then came back for a sabbatical leave from St. Louis University before moving to the University of Massachusetts Medical School in Worcester. While in Worcester, we started to collaborate again, and then we moved here to Boston University together in 1998. Associate Dean for Research Maria Kukuruzinska, another member of our department, was also a postdoctoral fellow in my group before coming to Boston University. John Samuelson and I started collaborating while I was still at

MIT and he was on the faculty of the Harvard School of Public Health, and his move to our group seemed like the most natural thing that could happen. Two other great colleagues are Miklos Sahin-Toth, who has made outstanding contributions to the field of hereditary pancreatitis, and David Levin, a true expert in signal transduction. Concerning family, I will just quote another of my idols, Gerald Fink, who wrote in his "Reflections" article, "Our spouses, often not scientists themselves, provide the connection between our scientific devotion and everyday life. They are companions in our success and balm in our failures." We can never thank them enough.

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